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13. ABSTRACT (Maximum 200 Words)

A strong inverse relationship between vitamin A and cancer development has been established. Vitamin A deficiency in experimental animals has been associated with a higher incidence of cancer and with increased susceptibility to chemical carcinogens. All-trans-retinoic acid (RA), an active form of vitamin A, has been used in both chemotherapy and chemoprevention of human cancers. We isolated a 1,745-nucleotide cDNA for a gene encoding a transcription factor, Rex-1 (for reduced expression), which is expressed at high levels in F9 teratocarcinoma stem cells. Its expression is rapidly reduced upon RA treatment of F9 cells. Furthermore, Rex-1 has been used as a marker for many types of stem cells. Since the Rex-1 gene encodes a transcription factor, we searched for Rex-1 target genes in order to understand the functions of Rex-1. We had previously made an F9 Rex-1$^{-/-}$ cell line by homologous recombination. We employed Affymetrix microarrays, using total RNA isolated from F9 WT and F9 Rex-1$^{-/-}$ cells, treated +/- RA, to search for Rex-1 target genes. Many putative Rex-1 target genes were identified, including transcription regulators, apoptosis factors, tumor suppressors, growth factors, signaling factors, and hormone receptors, which play important roles in cancer development. One of the Rex-1 target genes is the suppressor of cytokine signaling-3 (SOCS-3). The expression of the SOCS-3 gene is elevated in various breast carcinomas. We are now carrying out detailed studies of SOCS-3 to understand the mechanism of action of Rex-1 in RA induced stem cell differentiation and mammary epithelial cell differentiation. This research will contribute to the understanding of various types of cancer, and should lead to improved cancer therapies.

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Introduction

Retinoids (vitamin A and related compounds), important regulators of cell proliferation and differentiation, are used clinically in the treatment of various skin conditions and in cancer therapies. How retinoids cause cell differentiation is one focus of the laboratory. The Rex-1 gene (Fig. 1) was identified by a subtractive hybridization screen as a gene that was transcriptionally down-regulated by retinoids. The Rex-1 gene encodes a protein of 32.3 kilodaltons that contains four copies of a cys-his zinc finger motif, suggesting that Rex-1 may function as a transcriptional regulatory protein. Rex-1 protein is involved in both embryonic development, spermatogenesis, and is expressed at high levels in F9 teratocarcinoma cells and embryonic stem (ES) cells.
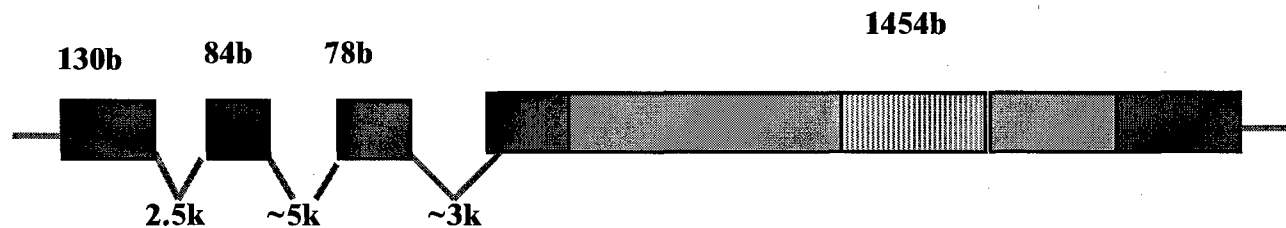

Fig. 1. Rex-1 (*Zfp-42*) Genomic Structure (1).
The Rex-1 gene is diagrammed (not to scale), showing exon-intron boundaries. Exons 1 through 4 are shown as open rectangles. Sizes of the exons (in base pairs) are indicated above the diagram. The ORF is contained within exon 4 and is shown in red rectangle. Approximate sizes of introns (in kilobases) are given below the diagram. The shaded red rectangle indicates the zinc finger region.

Teratocarcinomas of mice contain tumorigenic stem cells that can proliferate indefinitely in culture; these stem cells resemble the pluripotent embryonic stem cells of the inner cell mass of the blastocyst. The F9 murine teratocarcinoma stem cell line in monolayer culture differentiates into a homogeneous population of primitive endoderm cells in the presence of physiological concentrations of retinoic acid (RA) and into parietal endoderm cells in the presence of RA and dibutyryl cyclic AMP (cAMP). Alternatively, RA treatment of F9 stem cells grown as aggregates in suspension induces differentiation of the outer layer of cells into visceral endoderm. Parietal and visceral endoderm cells are extraembryonic epithelial cell types formed from the inner cell mass of the blastocyst during early mouse embryogenesis.

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Due to its potential function as a transcription factor in pluripotent stem cells and its developmentally restricted expression pattern, we examined the functions of Rex-1 in stem cell differentiation using F9 cells as a model system. Homologous recombination was used to disrupt both alleles of the Rex-1 gene in F9 cells (Fig. 2). Differentiation into parietal endoderm cells occurred with RA alone in the cells. However, in the presence of RA alone neither differentiation of the Rex-1^{-/-} cells into primitive endoderm nor into visceral endoderm occurred. To further study the role of Rex-1, we employed subtractive hybridization and Affymetrix Microarray to isolate potential Rex-1 target genes using total RNA isolated from F9 WT and F9 Rex-1^{-/-} cells. One of these target genes was SOCS-3 (suppressor of cytokine signaling –3).
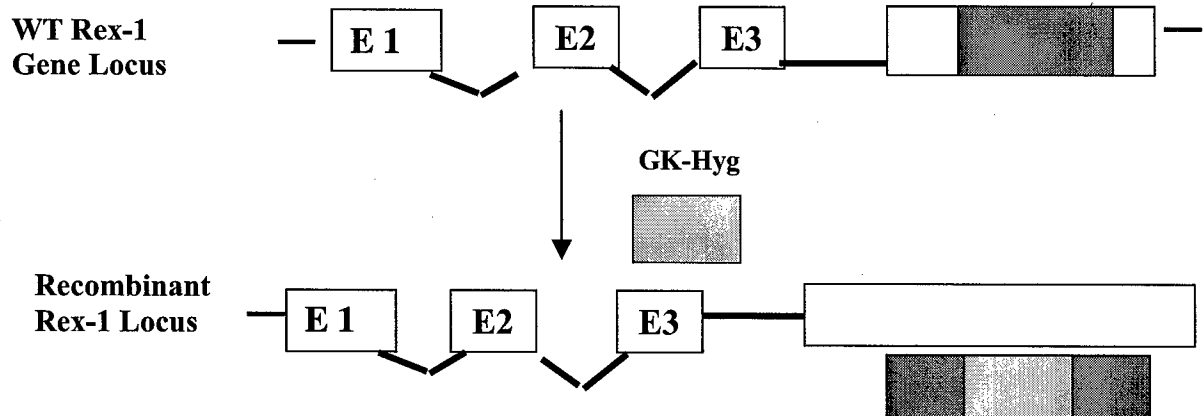

Fig. 2. Generation of a Targeted Deletion Construct of Rex-1 in F9 cells (2). A 3.4 kb BglII-HindIII fragment containing Rex-1 genomic sequence spanning 5' upstream sequence to codon 98 of the Rex-1 coding region, was subcloned into the BglII-HindIII sites of the vector pBSTK, a plasmid containing the thymidine kinase selection cassette in pBluescript. The pGKhygro cassette was subcloned into the 3' HindIII Rex-1genomic site and the flanking EcoRI site of the pBS vector.

Body

Identification of SOCS-3 as a Rex-1 Target Gene in F9 Cells by Microarray Analyses

I used DNA microarray assays to identify Rex-1 target genes. From the microarray experiments, the murine SOCS-3 gene scored the highest level of difference, showing a 17.7-fold higher expression in RA-treated R21 cells as compared to RA-treated F9 WT cells (Fig. 3) (Xu and Gudas, in preparation). This high level of difference in expression and the fact that SOCS-3 is a key regulator of the Stat3 signaling pathway resulted in my selection of the SOCS-3 gene for further analysis. Other Rex-1 putative target genes were p21 and p53 (Fig. 4).
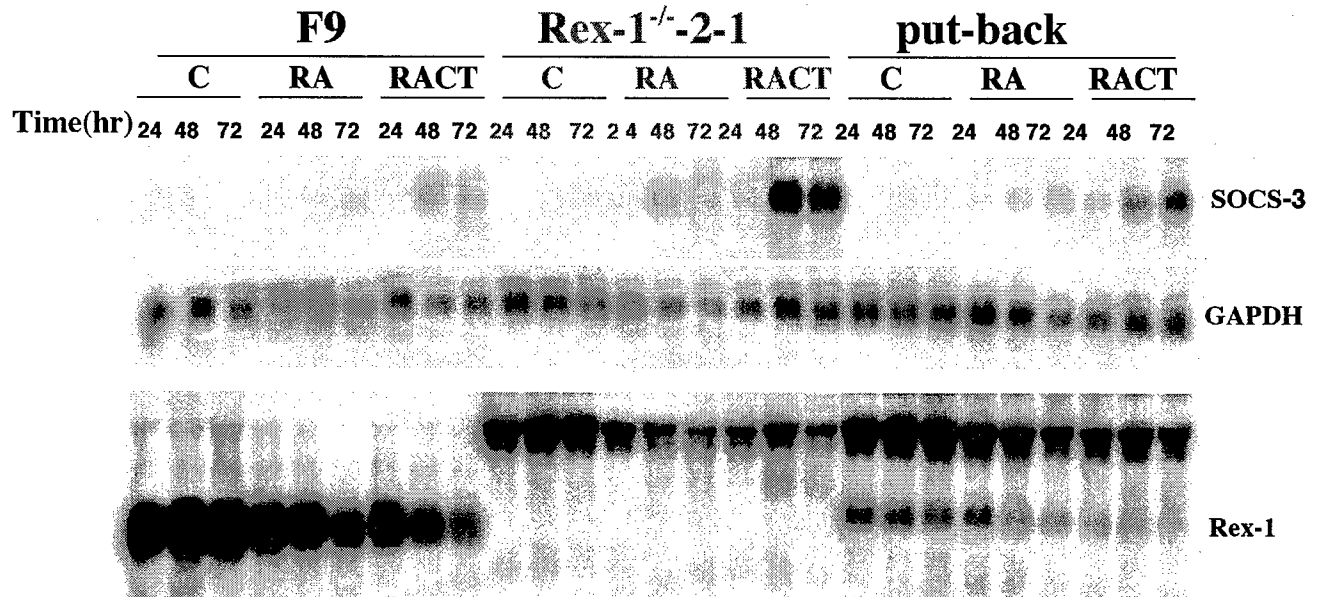

Fig. 3. **Northern Analyses of a Rex-1 Target, Suppressor of Signaling –3 (SOCS-3), in F9WT, F9 Rex-1$^{-/-}$, and Rex-1-reintroduced Lines.** Cytokines are key regulators of mammary epithelial cell function, exerting regulatory actions on proliferation, differentiation. Apoptosis, and immune surveillance. SOCS-3 is a STAT3-inducible feedback suppressors of STAT3 signaling. The overexpression of SOCS-3 decrease cell sensitivity to cytokines. The expression of SOCS-3 gene was examined in various breast carcinomas ands breast cancer lines, in comparison with normal tissue and breast lines (3). The elevated expression of SOCS-3 proteins within *in situ* ductal carcinomas relative to to normal breast tissue was reported. Significantly increased expression of SOCS-3 transcripts was also shown by RT-PCR in 10 cancer lines, but not in control lines. In my studies, the expression of SOCS-3 transcript was elevated in Rex-1$^{-/-}$ cells compared with F9 WT cells after RA, CT, amd RACT treatments. This implies that Rex-1 regulates stem cell differentiation though STAT3/SOCS-3 pcytokine signaling pathway and a similar pathway could also exist in the development of breast carcinoma.
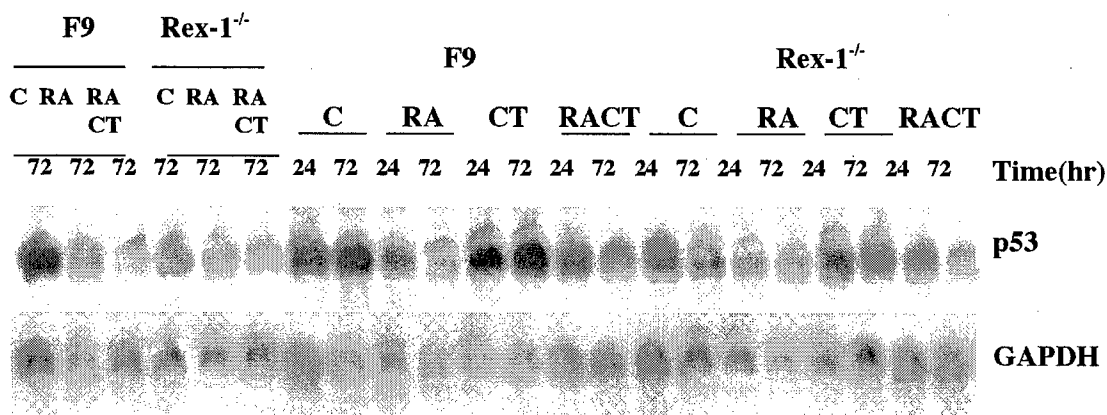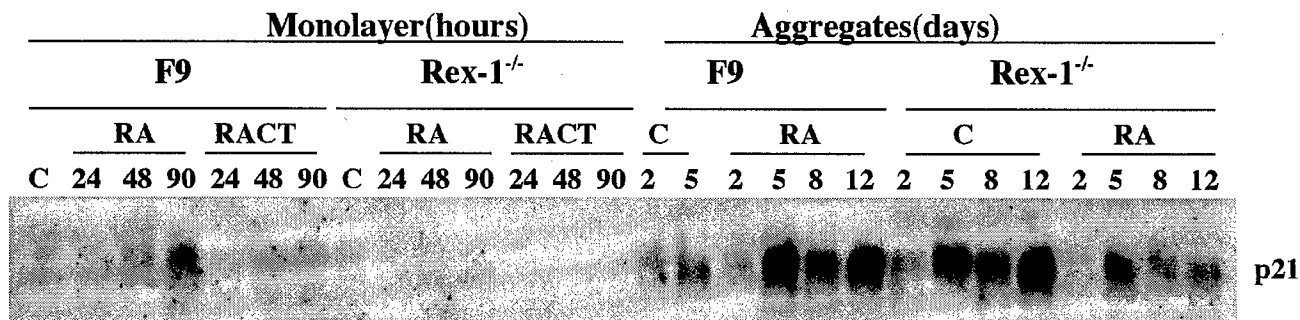
	Monolayer(hours)													Aggregates(days)											
	F9						Rex-1^{−/−}						F9							Rex-1^{−/−}						
		RA			RACT			RA			RACT		C		RA				C				RA			
C	24	48	90	24	48	90	C	24	48	90	24	48	90	2	5	2	5	8	12	2	5	8	12	2	5	8	12

p21

F9			Rex-1^{−/−}																				
C	RA	RA CT	C	RA	RA CT		F9								Rex-1^{−/−}								
						C		RA		CT		RACT		C		RA		CT		RACT			
72	72	72	72	72	72	24	72	24	72	24	72	24	72	24	72	24	72	24	72	24	72	Time(hr)

p53

GAPDH

Fig. 4. Northern Analyses of p21 and p53 in F9 WT and Rex-1^{−/−} Cells. P21 is a cell cycle inhibitor and p53 is a tumor suppresor. The expressions of both p21 and p53 have been shown to be lower in both breast cancer tissues and breast cancaer lines compared with normal breast tissue and normal breast epithelial lines. In my studies, I have shown that the expression of both transcripts are lower in Rex-1^{−/−} cells compared with their expressions in F9 WT cells. This result suggests that Rex-1 plays a role in the development of breast carcinomas.

Induction of the SOCS-3 gene expression by RA, CT, and RACT

We obtained a cDNA for murine SOCS-3 from Dr. James Ihle at St. Jude Children's Research Hospital, sequenced this to confirm that it was SOCS-3, and confirmed the data from microarray analysis by Northern analysis (Figure 3). F9 WT cells and two independently isolated Rex-1^{−/−} knockout cell lines (R21 and R5) were cultured for 24 h, 48 h, or 72 h in the presence of 1 μM RA, 250 μM db cAMP, and 250 μM theophylline (RACT) as previously described (4-8). In F9 WT cells, barely detectable levels of SOCS-3 mRNA were present in untreated cells, and RA or CT alone had very minimal effects on the expression of SOCS-3 transcripts at all the time points (Figure 3). The combination of RA and CT (RACT) induced the expression of SOCS-3 transcripts (for 48 h: 2.43 ± 0.58 fold, n=3, p= 0.0038; for 72 h: 5.74 ± 0.53 fold, n=3, p= 0.0004).

In F9 Rex-1$^{-/-}$ (R21) cells, the expression of SOCS-3 transcripts was also barely above a detectable level in untreated cells. After 48 h of RA addition, SOCS-3 mRNA expression was induced (for 48 h: 5.78 ± 0.83 fold, n=3, p= 0.035; for 72 h: 4.3 ± 0.53 fold, n=3, p= 0.005) (Figure 3.1). CT treatment alone also induced SOCS-3 expression (for 24 h: 2.32 ± 0.06 fold, n=3, p= 0.023; for 72 h: 3.32 ± 0.22 fold, n=3, p= 0.003), although the effect of CT was earlier and smaller than the effect of RA on SOCS-3 transcription. RACT treatment was able to induce SOCS-3 mRNA expression in Rex-1$^{-/-}$ cells to a much higher level than in F9 WT cells (for 24 h: 6.54 ± 1.83 fold, n=3, p= 0.021; for 48 h: 25.95 ± 3.53 fold, n=3, p= 0.001; for 72 h: 16.56 ± 0.78 fold, n=3, p= 0.0005). The same results were observed in two independently isolated F9 Rex-1$^{-/-}$ null cell lines R21 and R5) (Figure 3).

To confirm that the increase in SOCS-3 gene activation resulted from the absence of Rex-1$^{-/-}$, Rex-1 was stably transfected back into the F9 Rex-1$^{-/-}$ (R21) cells. In part because the exogenous Rex-1 gene was driven by a SV40 promoter and SV40-driven transcription is not efficient in F9 cells (9), the expression of Rex-1 in the Rex-1 put-back lines was about only 10% of its expression in F9 WT cells. This low level of Rex-1 mRNA was sufficient to partially reverse the expression pattern of SOCS-3 to its expression pattern in F9 WT cells (Figure 3), suggesting that a low level of Rex-1 expression is sufficient to inhibit SOCS-3 gene induction.

Induction of the SOCS-3 protein expression by RA, CT, and RACT

We also examined SOCS-3 protein levels in F9 WT, F9 Rex-1$^{-/-}$ (R21 and R5), and Rex-1 "put-back" (R21JX12) cells. Cells from each cell line were cultured in the presence of 1 μM RA, 250 μM db cAMP, and 250 μM theophylline (RACT) as previously described (4-8). After 72 h, total proteins were harvested for Western analysis of SOCS-3. In F9 WT cells, SOCS-3 protein was not expressed in control, RA-treated, and CT-treated cells (not shown). The treatment of RACT was able to induce SOCS-3 protein expression to a limited extent (<2 fold). In the two independently-isolated F9 Rex-1$^{-/-}$ null cell lines, SOCS-3 protein was not expressed in control cells. The treatment of RA or CT alone did not have any effect on SOCS-3 protein expression, while RACT greatly increased the SOCS-3 protein

level. In the cells in which Rex-1 was stably transfected back into the F9 Rex-1$^{-/-}$ (R21) cells, the expression pattern of the SOCS-3 protein was partially restored to its expression pattern in F9 WT cells.

RA, CT, and RACT activate the SOCS-3 promoter/reporter constructs; this activation is greatly augmented in the absence of Rex-1.

Clones 2, 6 and 6D2C (referred as 6D2 in the original paper) (10) were kindly provided to us by Dr. S. Melmed at Cedar-Sinai Research Institute. For measurement of SOCS-3 promoter activity, the F9 WT cells, the two F9 Rex-1$^{-/-}$ null lines (R21, R5), and the Rex-1 "put-back" line (R21JX12) were transiently transfected with pGL3Basic alone, clone 2 (nucleotides –2757 to –714), clone 6 (nucleotides –2757 to +929), or clone 6D2C (nucleotides –2757 to +929, with the complete tandem Stat binding region from nucleotides –99 to –60 deleted) (Figure 5). The vehicle, pGL3Basic, was used as a negative control. Clone 2 did not contain the minimal promoter region of SOCS-3 and hence it served as another negative control. The transfected cells were either untreated or treated with 1 μM RA, 250 μM db cAMP, and 250 μM theophylline (RACT) for 48 h. Then cells were harvested for luciferase assays. The durations of the treatments were determined based on the Northern results (Figure 3) in which the expression of SOCS-3 transcript was not induced until after 48 h of RA treatment of F9 Rex-1$^{-/-}$ cells.

In F9 WT cells, the effects of RA, CT, and RACT on clone 6 were not significant (for all values: fold change <2, n=4, p>0.05). The deletion of the Stat3 binding repeats did not alter the response of SOCS-3 promoter to RA, CT, and RACT treatments significantly (for all values: fold change <2, n=4, p>0.05).
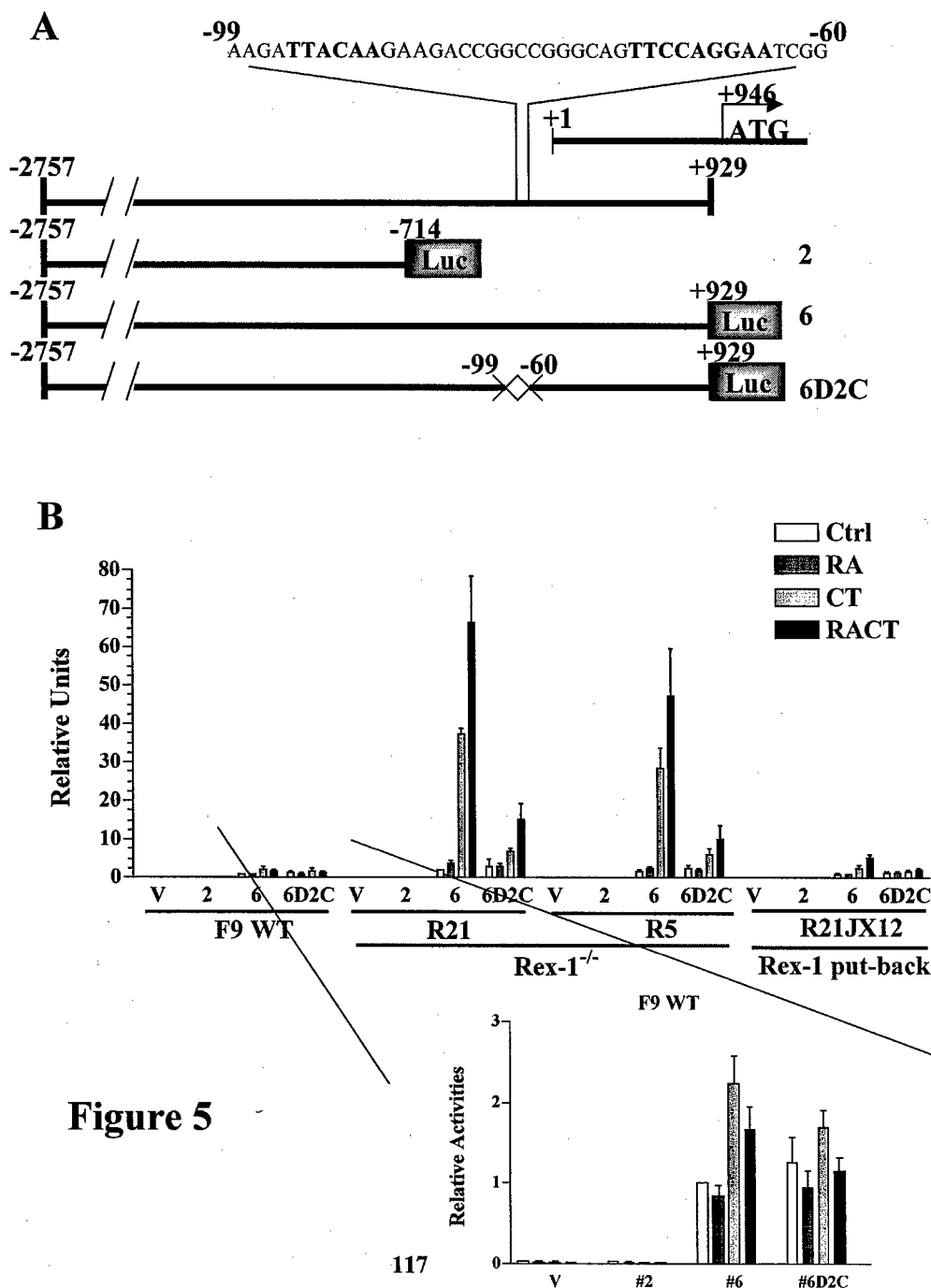
Fig. 5. Induction of the SOCS-3 promoter/reporter activity in F9 WT and F9 Rex-1$^{-/-}$ cells in response to RA, CT, and RACT. (A) Graphic representation of the ≈3.7 kb genomic 5' flanking region of the murine SOCS-3 promoter. The tandem Stat3 binding sites (in bold) and the start codon ATG are shown on the top. Diagrams of each pGL3 Basic-derived construct linked to the firefly luciferase reporter gene with different forms of the mouse SOCS-3 promoter are presented. The numbers refer to the 5' and 3' terminal nucleotides included in each construct with respect to the transcription start site of the above sequence. (B) F9 WT, R21, R5, and R21JX12 cells were transiently transfected with 4 μg of pGL3 Basic, clone 2, clone 6, or clone 6D2C. Each cell line was also co-transfected with 1.5 μg of a Renilla luciferase plasmid as a transfection control. Sixteen hours after the transfection, fresh medium was replaced in the presence of 1 μM RA, 250 μM db cAMP, and 250 μM theophylline (RACT). After 48 h of treatment, cells were harvested for luciferase assay. Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity. Relative luciferase activity was calculated as a ratio over nonstimulated F9 WT cells transfected with clone 6. Mean values with error bars as indicated are from four independent experiments.

In F9 Rex-1$^{-/-}$ (R21) cells, RA addition activated the SOCS-3 promoter (clone 6) by at most two-fold (2 ± 0.19 fold, n=4, p=0.05). The addition of CT was able to induce SOCS-3 promoter activity greatly (18.2 ± 2.5 fold, n=4, p=0.000015). The induction of the SOCS-3 promoter by RACT was even greater (34.4 ± 7.8 fold, n=4, p=1.5 x 10^{-6}). When the Stat3 binding repeats were deleted, the effect of RA on the promoter/reporter activity was no longer significant (1.2 ± 0.8 fold, n=4, p>0.05); the effect of CT on SOCS-3 promoter activity was no longer significant (3.2 ± 1.1 fold, n=4, p>0.05); the effect of RACT on SOCS-3 promoter activity was reduced to an activation of only 8 ± 2.7 fold (n=4, p=0.02), which was 6 ± 2.5 fold lower than RACT induction of clone 6 (34.4 ± 7.8 fold). Similar results were observed in the F9 Rex-1$^{-/-}$ (R5) cells. The activities of the SOCS-3 promoter constructs in the Rex-1 "put-back" cells were similar to their activities in F9 WT cells (Figure 5). We conclude that activated STAT-3 in the Rex-1$^{-/-}$ cells is responsible for the large increase in SOCS-3 promoter activity observed in these knockout cells.

Rex-1 in Human Breast Cancer

We then examined several cultured human breast cancer cell lines for Rex-1 expression, and observed expression in some of the lines (not shown, in preparation). One of these is MDA-MB-468. These data suggest that Rex-1 may be expressed in more undifferentiated, more malignant human breast cancers.

Research Accomplishments

- Identified gene targets of the Rex-1 transcription factor, including SOCS-3, p21, and p53.

- Demonstrated that SOCS-3 is highly induced in Rex-1$^{-/-}$ knockout cells after retinoic acid addition.

- Demonstrated that the SOCS-3 gene is regulated at the transcriptional level via Rex-1, and that an intermediary in this regulation is phospho-STAT-3.

- Demonstrated by RT-PCR and Western analysis that Rex-1 is highly expressed, at both the mRNA and protein levels, in some human breast cancers. One line which highly expresses Rex-1 is MDA-MB-468.

Reportable Outcomes

Xu, J., Sylvester, R., Chen, S., Tighe, A., and Gudas, L.J. SOCS-3 is a Rex-1 Target Gene. (2004) In preparation.

Xu, J. and Gudas, L.J. The p53 Gene is Transcriptionally Regulated by Rex-1. (2004) In preparation.

Conclusions

We've shown that Rex-1 encodes a transcription factor which negatively regulates a number of genes involved in cell differentiation. One of these genes, SOCS-3, was analyzed in depth. We showed that SOCS-3 is transcriptionally induced by Rex-1 via a STAT-3 binding site in the SOCS-3 promoter. Moreover, we demonstrated that both SOCS-3 and Rex-1 are highly expressed in some malignant human breast cancer lines.

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